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Predominance of *Trypanosoma cruzi* Lineage I in Mexico

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Randomly amplified polymorphic DNA (RAPD) has emerged as an effective genetic marker for analysis of *Trypanosoma cruzi* population variability. This method has been used to study the genetic variability of Mexican *T. cruzi* stocks and to relate these results to previous classifications. High clonal diversity was observed among the Mexican populations: 24 RAPD types were scored among 56 stocks analyzed. Only two stocks (3.6%) belonged to the *T. cruzi* II lineage, while all others belonged to *T. cruzi* I. The robustness of these clusters was statistically highly significant. Mexican *T. cruzi* I stocks formed a homogeneous group with reduced genetic distances among its members. Parasites from this group were isolated from both domestic and sylvatic cycles over a broad geographic area in Mexico. The two Mexican stocks classified as *T. cruzi* II (isolated from sylvatic cycles) were of the same RAPD type, although they were not closely related to the three reference *T. cruzi* II stocks circulating in domestic cycles in Argentina, Brazil, Bolivia, and Chile. These stocks were also unrelated to the formerly named Zymodeme III.

Trypanosoma cruzi, the agent of Chagas' disease, which is widely distributed in Latin America, shows considerable genetic polymorphism (14). Extensive studies carried out by using multilocus enzyme electrophoresis have demonstrated that natural populations of *T. cruzi* have a clonal structure (21), which has led to subdivision of the taxon into two major lineages, *T. cruzi* I and *T. cruzi* II (1, 13, 18, 20). Each lineage is genetically heterogeneous, and despite failure to identify subdivisions within *T. cruzi* I, five subgroups of *T. cruzi* II have been proposed as discrete typing units (DTU) on the basis of randomly amplified polymorphic DNA (RAPD) analysis (1, 5). These six DTU have been proposed as a reference framework for genetic variability and for biological characterization studies of *T. cruzi* stocks.

A few studies have attempted to characterize Mexican *T. cruzi* stocks by genetic methods. Seventeen stocks were analyzed using *Hin*dIII restriction fragment length polymorphisms associated with rRNA gene spacers and kinetoplast DNA maxicircle polymorphisms (9, 12, 22). These studies identified two groups of Mexican stocks, which correlate with geographic origin, although assignment to a *T. cruzi* lineage was not possible. The same stocks were also analyzed by multilocus enzyme electrophoresis, demonstrating a high degree of heterogeneity that was not related to geographic origin (12). Analysis of minicircle kDNA variability among Mexican stocks showed no correlation between the sequence homologies of the hypervariable region of minicircles and clustering (2), contrasting with results obtained for South American stocks (3, 4).

In the present study, 56 Mexican stocks, isolated from diverse hosts and with diverse geographic origins, were analyzed by RAPD. Relevant phylogenetic analyses were used to assess population variability and structure, and to evaluate the taxonomic position of Mexican stocks and their correspondence with recent *T. cruzi* classifications.

MATERIALS AND METHODS

Origins of the stocks. A panel of 56 *T. cruzi* Mexican stocks was selected from 155 stabilized isolates. Table 1 summarizes the site (by state) and host origins of the stocks analyzed. Seven previously characterized reference stocks were also included in the analysis (Table 2). Parasites were bulk cultured in LIT liquid medium and harvested by centrifugation, and parasite pellets were stored at −70°C.

Typing by RAPD analysis. DNAs were extracted from cell pellets as previously reported (5), and their concentrations and purities were determined by spectrophotometry (at 260 and 280 nm) using 10-μl samples. Samples were aliquoted and stored at −20°C (10 μg/ml). PCRs were performed in 0.2-ml microcentrifuge tubes containing 60 μl of reaction mixture. The composition of the reaction mixture and the amplification conditions were identical to those previously described (5). Amplifications were performed on a PTC-100 programmable thermal cycler (MJ Research) programmed for 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C, followed by a final elongation step of 7 min at 72°C. Negative controls using DNA-free water as the template were added for each PCR procedure. Amplification products were separated by electrophoresis in a 2% agarose gel (in Tris-acetate-EDTA [TAE]) and were detected by staining with ethidium bromide. Data were obtained for the following 10 primers, which gave the most easily readable and consistent patterns (kit A; Operon Technology, Alameda, Calif.): OPA-02, OPA-04, OPA-08, OPA-09, OPA-13, OPA-14, OPA-16, OPA-17, OPA-19, and OPA-20.

Data analysis. The RAPD Distance Program, version 1.04 (available at ftp://life.anu.edu.au/pub/software/RAPDistance or http://life.anu.edu.au/molecular/software/rapd.html), was used to analyze RAPD bandings, while Jaccard's phenetic distances were used to differentiate between pairs of stocks. Relationships between genotypes were depicted using the UPGMA (unweighted pair group method with arithmetic average) and neighbor-joining methods with the NEIGHBOR program of the PHYLIP package, version 3.5c (Department of

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TABLE 1. Origins of Mexican *T. cruzi* stocks examined by RAPD

Stock	Host	Locality	State	RAPD numbering
ITRI/MX/98/Col-08	*Triatoma longipennis*	Nogueras	Colima	R15
ITRI/MX/98/Col-18	*T. longipennis*	Nogueras	Colima	R05
ITRI/MX/98/Col-07	*Triatoma pallidipennis*	Nogueras	Colima	R01
MHOM/MX/97/Inc-5	Human		Guanajuato	R09
MHOM/MX/95/JRA	Human		Jalisco	R01
MHOM/MX/93/JJO	Human		Jalisco	R13
ITRI/MX/98/Tep-61	*Triatoma barberi*	Tepehuaje	Jalisco	R09
ITRI/MX/98/Sma-230	*T. longipennis*	Ipalzoltic	Jalisco	R10
ITRI/MX/98/Sma-212	*T. longipennis*	San Martin de Hidalgo	Jalisco	R06
ITRI/MX/98/Tep-22	*T. longipennis*	Tepehuaje	Jalisco	R14
ITRI/MX/98/Tep-19	*T. longipennis*	Tepehuaje	Jalisco	R18
MDID/MX/97/Mor-11	*Didelphis virginiana*	Temixco	Morelos	R02
MHOM/MX/-/M93-5	Human	Cuernavaca	Morelos	R12
MHOM/MX/93/EA	Human		Morelos	R01
MHOM/MX/95/Mor-03	Human	Temixco	Morelos	R16
ITRI/MX/98/800296	*T. pallidipennis*	Chalcatzingo	Morelos	R02
ITRI/MX/98/800383	*T. pallidipennis*	Juitepec	Morelos	R05
ITRI/MX/-98800481	*T. pallidipennis*	Chalcatzingo	Morelos	R05
ITRI/MX/99/8005762	*T. pallidipennis*	Juitepec	Morelos	R06
ITRI/MX/98/800389	*T. pallidipennis*	Xochitepec	Morelos	R09
ITRI/MX/98/800295	*T. pallidipennis*	Chalcatzingo	Morelos	R05
ITRI/MX/98/800394	*T. pallidipennis*	Temixco	Morelos	R05
ITRI/MX/97/Mor-10	*T. pallidipennis*	Cuernavaca	Morelos	R07
ITRI/MX/98/800209	*T. pallidipennis*	Emilio Zapata	Morelos	R05
ITRI/MX/99/800682-6	*T. pallidipennis*	Huantla	Morelos	R02
ITRI/MX/99/Cari-035	*Triatoma phyllosoma*[a]	Carrillo Puerto	Nayarit	R03
ITRI/MX/98/Nay-016	*T. longipennis*	Puga	Nayarit	R01
ITRI/MX/99/Pla-23	*Triatoma phyllosoma*	Platanito	Nayarit	R03
ITRI/MX/98/Com-1	*Triatoma picturata*	Compostela	Nayarit	R01
ITRI/MX/99/Pla-20	*T. picturata*	Platanito	Nayarit	R03
ITRI/MX/99/Cari-145	*T. picturata*	Carrillo Puerto	Nayarit	R09
ITRI/MX/99/Cari-078	*T. picturata*	Carrillo Puerto	Nayarit	R03
ITRI/MX/99/Cari-006	*T. picturata*	Carrillo Puerto	Nayarit	R04
ITRI/MX/99/Cari-018	*T. longipennis*	Carrillo Puerto	Nayarit	R09
ITRI/MX/99/Cari-144	*T. picturata*	Carrillo Puerto	Nayarit	R01
MHOM/MX/94/Ninoa	Human		Oaxaca	R02
MHOM/MX/97/Inc-1	Human		Oaxaca	R09
MHOM/MX/93/FRV	Human		Oaxaca	R06
MHOM/MX/93/Cid	Human		Oaxaca	R04
ITRI/MX/98/Sba-026	*T. barberi*	San Bartolo de Coyotepec	Oaxaca	R01
ITRI/MX/98/Sba-056	*T. barberi*	San Bartolo de Coyotepec	Oaxaca	R01
ITRI/MX/98/Jva-022	*T. barberi*	Jalapa del Valle	Oaxaca	R09
ITRI/MX/98/Sba-35	*T. barberi*	San Bartolo de Coyotepec	Oaxaca	R03
MDID/MX/91/Ver-04	*Didelphis marsupialis*		Veracruz	R06
MDID/MX/91/Ver-06	*D. virginiana*		Veracruz	R20
MPHI/MX/89/Ver-05	*Philander oppossum*		Veracruz	R01
MPHI/MX/91/Ver-03	*P. oppossum*		Veracruz	R20
MDID/MX/97/Z-44	*Didelphis* sp.		Yucatán	R08
MDID/MX/98/Z-56	*Didelphis* sp.		Yucatán	R11
MHOM/MX/93/H1	Human		Yucatán	R01
ITRI/MX/98/Cux-24	*Triatoma phyllosoma* sp.[a]	Cuxpala	Zacatecas	R05
MHOM/MX/93/Zac	Human		Zacatecas	R01
ITRI/MX/99/Cux-50	*T. longipennis*	Cuxpala	Zacatecas	R01
ITRI/MX/98/Cux-21	*T. longipennis*	Cuxpala	Zacatecas	R10
ITRI/MX/98/Cux-46	*T. longipennis*	Cuxpala	Zacatecas	R01
ITRI/MX/99/Cux-36	*T. longipennis*	Cuxpala	Zacatecas	R01

[a] Instar belonging to the *T. phyllosoma* complex.

TABLE 2. Origins of *T. cruzi* reference stocks

Stock	Host	Country	Reference	RAPD numbering
MHOM/BR/78?/Sylvio-X10-cl1	Human	Brazil (Belém)	13	R01
MHOM/BR/77/Esmeraldo-cl3	Human	Brazil (Bahia)	13	R23
MHOM/BR/68/CANIII-cl1	Human	Brazil (Belém)	13	R24
MHOM/CL/-/CBB	Human	Chile (Tulahuén)	15	R22
ITRI/BO/86/SO34-cl4	*Triatoma infestans*	Bolivia (Potosi)	5	R17
ITRI/BO/93/MIZ 03	*T. infestans*	Bolivia (Cochabamba)	4	R21
ITRI/BO/92/CAR 30	*T. infestans*	Bolivia (La Paz)	4	R19

Genetics, University of Washington, Seattle). Wagner's parsimony and node robustness were evaluated by bootstrap analysis (7) using Seqboot, Mix, and Consense (PHYLIP package) software. Trees were drawn with the TREEVIEW program (15). A multiple correspondence analysis (MCA) of the same data was performed using the ADE-4 program (19).

RESULTS

RAPD variability. The 56 Mexican stocks were analyzed with 10 individual primers by RAPD and compared with the 7 reference stocks (Tables 1 and 2). All amplifications were repeated at least twice, confirming the reproducibility of the most intense bands. Some low-intensity bands were not reproducible and therefore were not included in the comparative analysis. Electrophoresis profiles of the amplified products for each primer were composed of 1 to 16 DNA fragments ranging in size from 0.2 to 2.5 kb. In total, 66 different DNA fragments were detected and 22 to 30 bands were generated for each isolate. Twenty-four RAPD types, based on specific band patterns, were identified among all stocks (Table 1). Six of the reference stocks had individual RAPD types not shared with Mexican stocks. Mexican stocks expressed 18 RAPD types; 14 of the Mexican stocks had the R01 RAPD type, the same as that of the reference stock Sylvio-X10-cl1. Among the Mexican stocks, individual RAPD patterns were expressed either in isolated stocks (1 or 2) or in large numbers (e.g., R01 was expressed in 14 stocks [Table 1]). Three classes of bands were observed (Fig. 1): invariant bands (present in all RAPD types), singleton bands (present in only one RAPD type), and parsimony bands (common to multiple RAPD types). Among Mexican and reference stocks, six invariant bands were identified (9.1%). These were generated using primers A08 and A20 (1 constant band each), A02 (1 band of 2 generated with this primer), A04 (2 bands of 12), and A13 (1 band of 16). Nineteen bands were singleton (29%), and among these, four were observed in Mexican stocks (R12, R13, R14, and R20 [Table 1]). The majority of the 46 bands identified in the Mexican stocks (62%) were common to a large number of RAPD types. Ten of these were invariant bands (22%); 14 were singleton bands (30%), of which 9 were present in RAPD type R20; and the majority of the 22 common bands were present in 17 of the 18 RAPD types (Fig. 2).

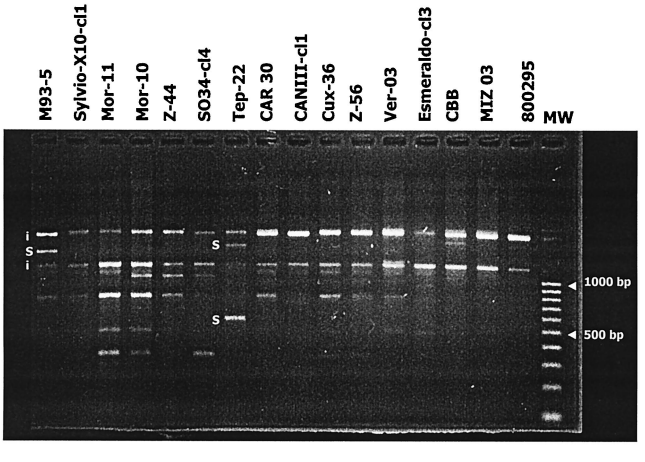

FIG. 1. RAPD patterns obtained with primer OPA-04 for different *T. cruzi* stocks. Total genomic DNA of each stock was amplified by PCR. Amplification products were analyzed by electrophoresis in 2% agarose gels and were detected by staining with ethidium bromide. MW, molecular weight markers; i, invariant bands; s, singleton bands. Unmarked bands are parsimony bands.

Clustering analysis. Jaccard's distances between pairs of RAPD types were calculated, and the distance matrices were transformed into unrooted trees by using either the neighbor-joining or the UPGMA method. Similar branching patterns were obtained by the two methods (Fig. 2). The majority of stocks clustered into a first group with minor variability. This group included two reference stocks (SO34-cl4 and Sylvio-X10-cl1) belonging to *T. cruzi* I. The second group was more heterogeneous, with three reference stocks of *T. cruzi* II (Esmeraldo-cl3, CBB, and MIZ 03) and two Mexican stocks isolated from sylvatic mammals. The CAR 30 and CANIII-cl1 reference stocks were distant from the previous two groups and were subsequently used as an outgroup for Wagner parsimony analysis (cladistic method). The Wagner tree confirmed the two principal clusters, and the bootstrap values obtained at the nodes were highly significant in both cases (98 and 92% for *T. cruzi* I and *T. cruzi* II, respectively [see Fig. 2]). In the absence of a more pertinent outgroup (CAR 30 and CANIII-cl1 belong to the *T. cruzi* taxon), it was impossible to assign CAR 30 and CANIII-cl1 to either *T. cruzi* I or *T. cruzi* II by cluster analysis. Hence, MCA was used with the 66 variables to test CAR 30 and CANIII-cl1 assignment to *T. cruzi* I or II (Fig. 3). The two first axes explained 47.6% of the complete variability, and statistical analysis confirmed with a significant absence of overlap that the stocks attributed to *T. cruzi* I and *T. cruzi* II (see above) are distinct groups ($P = 0.01$ for the MCA performed without CAR 30 and CANIII-cl1). The MCA analysis including CAR 30 and CANIII-cl1 stocks demonstrated that these stocks belonged to *T. cruzi* II ($P = 0.1$) and not to *T. cruzi* I ($P = 0.1$).

Ver 03 and Ver 06 stocks had banding patterns distinct from those of other Mexican stocks and clustered specifically with *T. cruzi* II by all analyses. Nevertheless, they were also signifi-

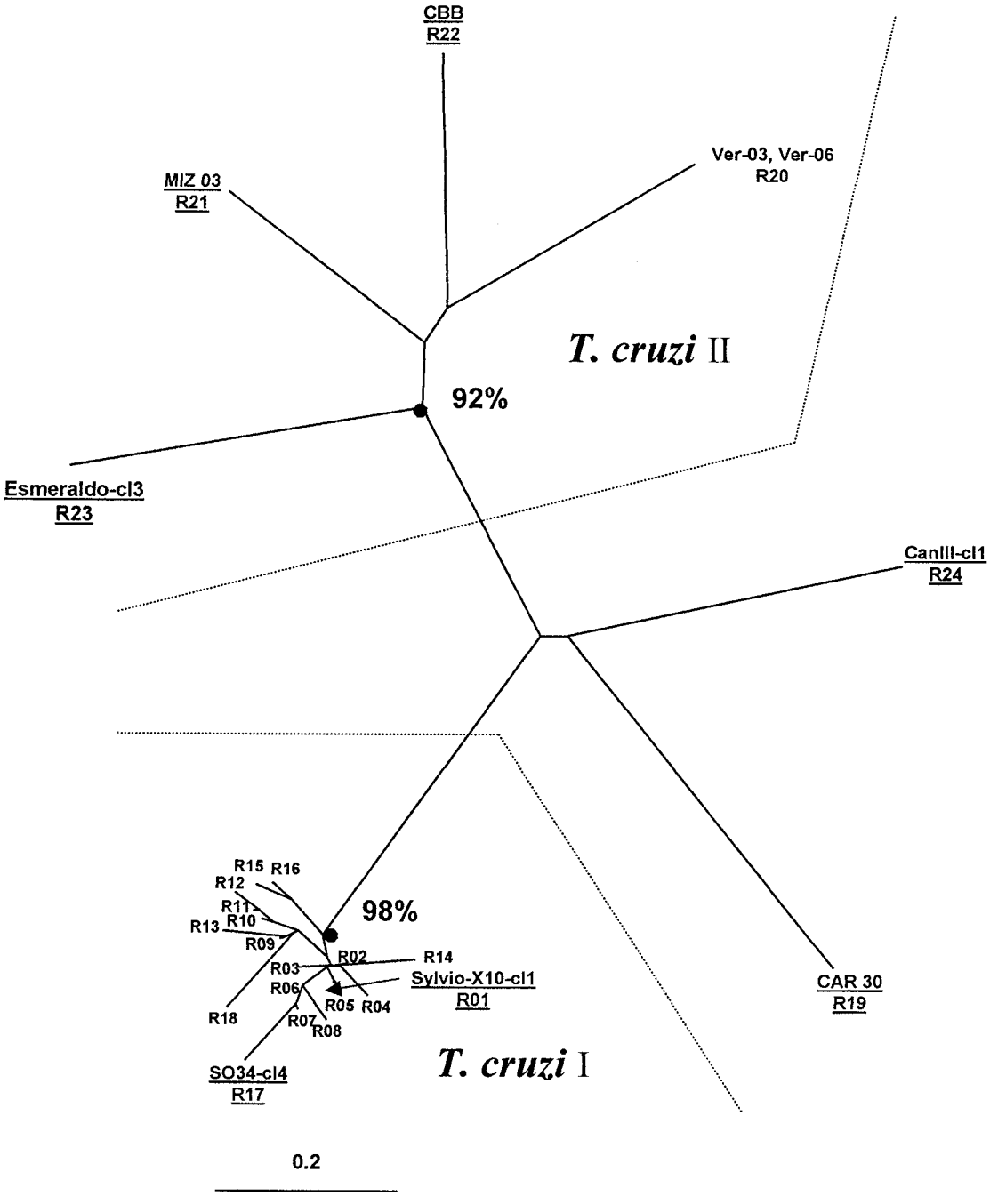

FIG. 2. Unrooted neighbor-joining tree derived from Jaccard's genetic distances calculated among the 24 different RAPD types. The different genotypes are localized at each terminal branch. Reference stocks are underlined. *T. cruzi* I is composed of genotypes R01 to R18, and *T. cruzi* II comprises genotypes R20 to R23. Only significant bootstrap values, obtained by Wagner parsimony analysis, are indicated for the principal nodes.

cantly distinct from other members of *T. cruzi* II (average Jaccard's genetic distance from other members of *T. cruzi* II, 0.30 ± 0.02) and from the former Zymodeme III group (CANIII-cl1 [13]), with a Jaccard distance of 0.55.

DISCUSSION

Mexican stocks belong to *T. cruzi* I. *T. cruzi* is composed of natural clones which have broad biological heterogeneity. Despite this heterogeneity, previous population genetics studies have attempted to classify *T. cruzi* stocks into discrete phylogenetic subgroups. Two clear DTU corresponding to the formerly designated *T. cruzi* I and *T. cruzi* II have been identified, and the latter DTU has been additionally divided into five subdivisions by use of both isoenzyme and RAPD markers (1, 5). This classification should be considered a tentative basis for future genetic diversity and biological property studies of *T.*

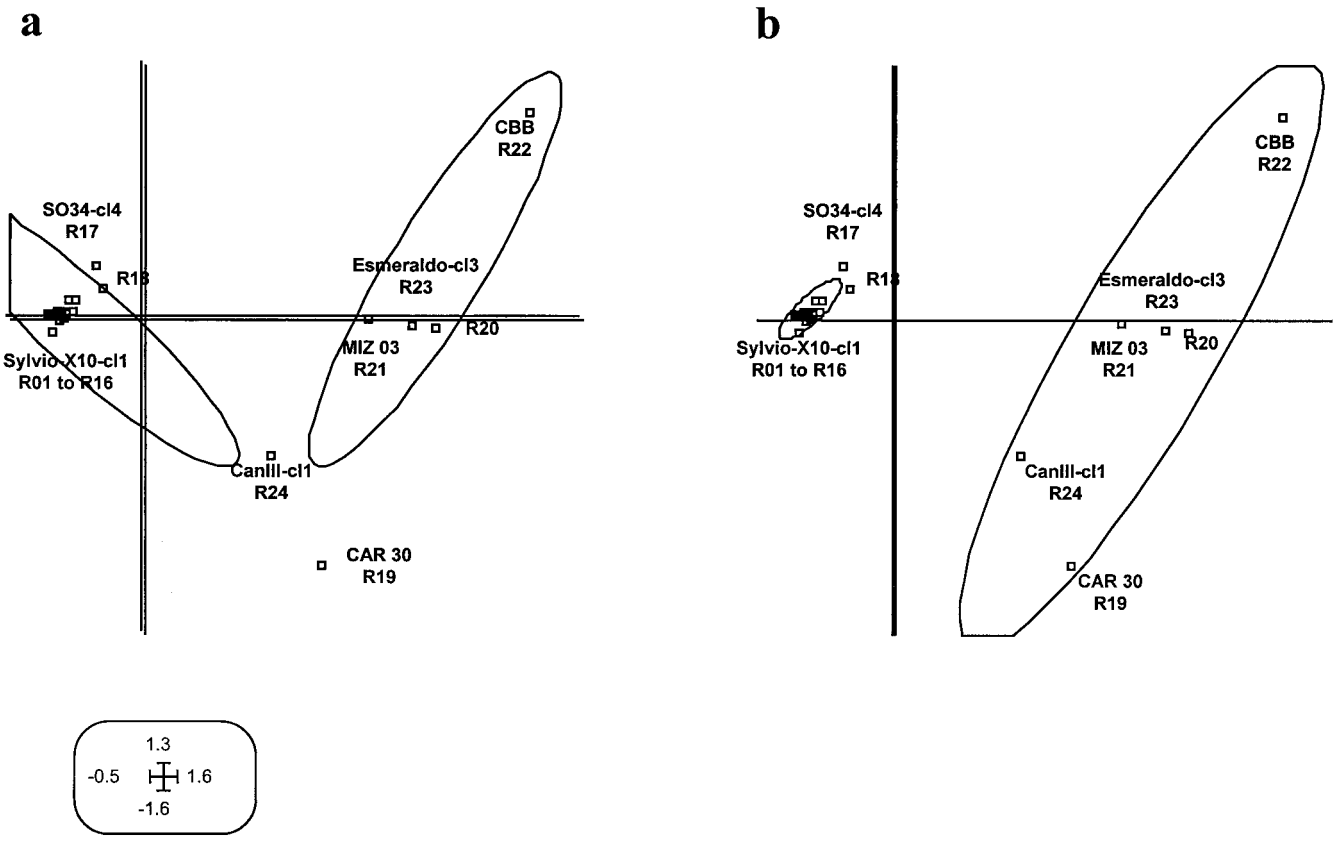

FIG. 3. MCA of the 24 different RAPD types scored. The ADE-4 ellipses option computes the means, variances, and covariance of each group of points on both axes and draws a corresponding ellipse. This ellipse theoretically clusters 90% of the points belonging to the different groups chosen. (a) CAR 30 and CANIII-cl1 stocks attributed to *T. cruzi* I; (b) CAR 30 and CANIII-cl1 stocks attributed to *T. cruzi* II.

cruzi. Although the biological diversity of these clusters remains unclear, several studies suggest that clonal diversity does have an impact on the biological behavior of *T. cruzi* stocks (10, 16, 17). In order to further study this correlation, information regarding the genetic diversity of stocks circulating within a country is essential.

In order to study the genetic diversity of *T. cruzi* populations in Mexico, a large sample of stocks isolated from eight states, four principal vector species, humans, and sylvatic mammals was selected. The results clearly demonstrate that most Mexican *T. cruzi* stocks (except for 2.4%) belong to the *T. cruzi* I lineage. Different statistical analyses cluster these isolates with two reference stocks belonging to *T. cruzi* I. Given the prevalence of seropositive individuals and of disease cases in the country, *T. cruzi* I stocks can be considered the primary agents of Chagas' disease in Mexico (8). The two Mexican stocks Ver 03 and Ver 06 were not closely related to the *T. cruzi* II reference stocks CBB, MIZ 03, and Esmeraldo-cl3, although they unequivocally clustered with them. This result contrasts with the situation in several South American countries, where parasites belonging to *T. cruzi* II play a major role in human infection. Furthermore, comparison of these stocks with the formerly described Zymodeme III, which is associated with sylvatic cycles, demonstrates that they are unrelated.

Mexican stocks belonging to *T. cruzi* I are closely related to each other. The lack of variability detected among the Mexican *T. cruzi* I stocks was notable compared to the total variability of the stocks studied. Of the 66 RAPD-scored bands, 41 were present in *T. cruzi* I Mexican stocks and 40% of them were invariant, while banding homology between pairs of stocks ranged from 86 to 99%. Furthermore, the absence of significant bootstrap values at the nodes precludes subdivision of this group by cladistic analysis (all <55%; average value, 20.4% ± 16%). The stock cluster into one group by using the MCA method (data not shown), indicating a clear absence of secondary structure. The average genetic distance (Jaccard's distances) calculated between pairs of Mexican *T. cruzi* I stocks (0.08 ± 0.04) was much lower than that with other *T. cruzi* I samples. A broad sample, representative of the geographic distribution of *T. cruzi* stocks tested, had an average Jaccard genetic distance of 0.36 ± 0.13 (2); with a reduced sample of stocks from sylvatic cycles isolated in French Guiana, the distance was similar, 0.36 ± 0.12 (11). Consequently, the reduced polymorphism among Mexican stocks is remarkable, given their broad geographic distribution, varied hosts, and isolation from domestic and sylvatic hosts. They belong to a single homogeneous group. Previous studies favored the possibility of broad biological variability among Mexican stocks. Espinoza et al. (6) described low- and high-virulence stocks (more than 80% of infected mice killed) in a murine model associated with different genotypes characterized by rRNA gene spacer polymorphism. All of these stocks can now be classified as *T. cruzi* I.

Therefore, genetic homogeneity by RAPD does not correlate with virulence heterogeneity. Further studies of pathogenic mechanisms will be necessary in order to better understand the biological implications of the microvariability among Mexican stocks.

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